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Molecular characterization of the VP4, VP6, VP7, and NSP4 genes of lapine rotaviruses identified in Italy: emergence of a novel VP4 genotype

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Abstract

The genes encoding the glycoprotein VP7, the VP8* trypsin-cleavage product of the protein VP4, a fragment of the protein VP6 associated with subgroup (SG) specificity, and the enterotoxin NSP4 of rotavirus strains identified in diarrheic fecal samples of rabbits in Italy were sequenced. The Italian lapine rotavirus (LRV) strains possessed a G3 VP7, SG I VP6, and KUN-like NSP4, a gene constellation typical of LRVs. One LRV strain (30/96), isolated in 1996, shared the closest amino acid (aa) identity (87–96%) with the P[14] genotype, composed of human and LRV strains. Conversely, three LRV strains (160/01, 229/01, and 308/01), identified in 2001, were highly identical (90–95%) among each other, but showed low aa identity (34–77%) to the VP8* genotype-specific sequences of representative rotavirus strains of all remaining P genotypes. This report confirms the worldwide genetic constellations of LRVs and identifies a novel VP4 genotype in rabbits, tentatively proposed as genotype P[22].

Keywords: Rotavirus; Diarrhoea; Rabbit; P genotype; VP4; VP6; VP7; NSP4

Introduction

Rotaviruses are the main cause of acute viral gastroenteritis in humans and animals throughout the world. Rotaviruses belong to the Reoviridae family and are characterized by a genome consisting of 11 segments of double-stranded RNA (dsRNA) enclosed in a triple-layered capsid (Estes, 2001). The inner capsid protein VP6 allows rotavirus classification into seven antigenically distinct serogroups (A to G) and bears the subgroup (SG) specificities that allow antigenic classification of group A rotaviruses into SG I, SG II, both SG I and II, or into neither SG based on reactivity with SG specific monoclonal antibodies (MAbs) 255/60 and 631/9 (Estes, 2001). Human rotaviruses (HRVs) belong mostly to SG II, whereas most animal rotaviruses belong to SG I (Iturriza-Gómara et al., 2002). The nonstructural glycoprotein NSP4 has been studied extensively because of its multiple functions in rotavirus morphogenesis and pathogenesis and its enterotoxic activity (Ball et al., 1996; Estes, 2001). Sequence analyses of the NSP4 gene from human and animal rotavirus strains has revealed the presence of four distinct NSP4 genogroups, KUN- (A), Wa- (B), AU-1- (C), and EW-like (D) (Horie et al., 1997; Kirkwood and Palombo, 1997; Cunliffe et al., 1997; Ciarlet et al., 2000), with a fifth, avianlike (E), NSP4 genogroup identified recently (Mori et al., 2002).

The two outer capsid proteins, VP4 and VP7, associated

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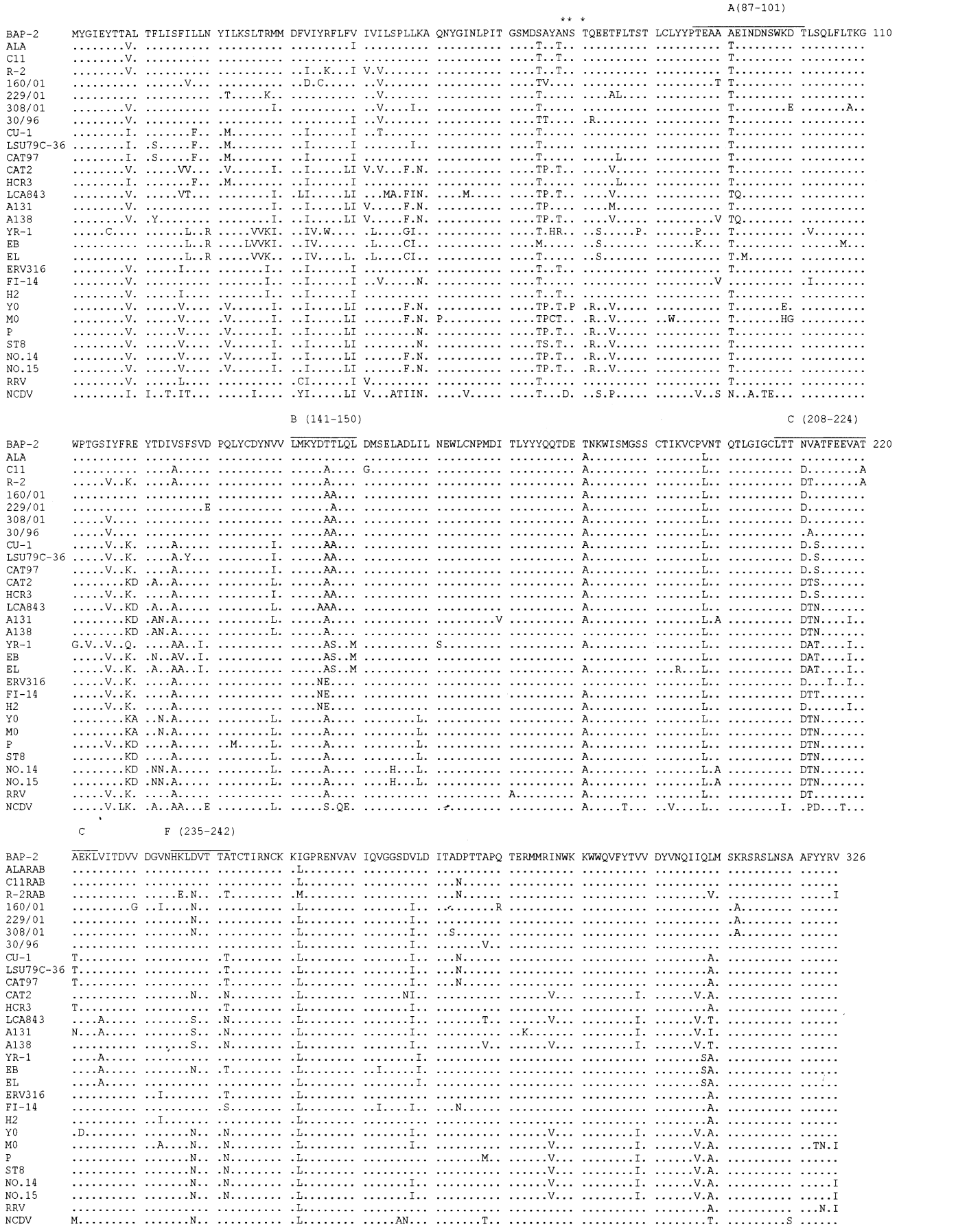

Fig. 1. Deduced amino acid sequence of the VP7 protein of the Italian LRV strains 30/96, 160/01, 229/01, and 308/01 and of a selection of G3 rotavirus strains of different species of origin. The VP7 antigenic regions A, B, C, and F are indicated. The glycosylation site NST (aa 69–71) is indicated by asterisks (*). Accession numbers of the VP7 sequences are listed in Table 1.

Table 1
Amino acid sequence identity of VP7 of Italian lapine strains to rotaviruses[a] belonging to various G serotypes

Strain (origin)	VP7 serotype	160/01	229/01	308/01	30/96
KU (human)	1	81.2	80.5	81.6	82.2
S2 (human)	2	73.8	74.1	74.6	74.9
YO (human)	3	88.6	89.2	89.6	90.5
RRV (simian)	3	92.0	92.3	93.2	94.5
Bap-2 (lapine)	3	93.8	95.1	94.2	95.1
Ala (lapine)	3	94.4	95.4	95.4	96.9
C11 (lapine)	3	94.1	94.7	95.1	95.4
R-2 (lapine)	3	91.6	91.0	92.9	93.6
160/01 (lapine)[b]	**3**	—	**95.7**	**95.4**	**94.7**
229/01 (lapine)[b]	**3**	**95.7**	—	**96.0**	**95.1**
308/01 (lapine)[b]	**3**	**95.4**	**96.0**	—	**96.0**
30/96 (lapine)[b]	**3**	**94.7**	**95.0**	**96.0**	—
ST3 (human)	4	77.2	77.8	78.5	78.5
OSU (porcine)	5	83.3	83.6	85.3	85.0
NCDV (bovine)	6	82.4	83.9	83.7	84.4
Ch2 (avian)	7	59.6	59.6	60.2	60.8
B37 (human)	8	79.9	79.9	80.7	80.7
116E (human)	9	81.2	81.2	83.1	84.4
61A (bovine)	10	81.2	80.9	82.5	83.7
YM (porcine)	11	85.8	85.8	87.1	87.7
L26 (human)	12	80.2	80.2	81.0	80.1
L338 (equine)	13	80.5	80.5	81.9	81.6
CH3 (equine)	14	83.3	84.2	85.0	85.3
Hg18 (bovine)	15	76.5	76.8	77.6	77.6

[a] GenBank accession nos. of VP7 genes: KU (D16343), S2 (M11164), YO, (D86284), RRV (AF295303), Bap-2 (U62153), ST3 (P10501), OSU (X04613), NCDV (M63266), Ch2 (X56784), B37 (J04334), 116E (L14072), 61A (X53403), YM (M23194), L26 (M58290), L338 (D13549), CH3 (D25229), Hg18 (AF237666), 160/01 (AF528202), 229/01 (AF528203), 308/01 (AF528201), and 30/96 (AF528204).

[b] Predicted G serotype on the basis of sequence similarity. The Italian lapine rotaviruses are in bold. The amino acid sequences of strains Ala, C11, and R-2 are by Nishikawa et al. (1989).

with P and G serotype specificity, respectively, independently elicit neutralizing antibodies and induce protective immunity (Estes, 2001). Classification of rotaviruses into VP4 or VP7 serotypes is performed by cross-neutralization assays using hyperimmune sera against VP4 alone or the whole virus, respectively. Based on either antigenic or genetic characterization of VP7, 15 G serotypes have been identified (Estes, 2001; Hoshino et al., 2002). Due to the lack of appropriate antibody reagents, P serotypes and P genotypes (defined by sequence analysis and/or nucleic acid hybridization data and designated in brackets) are used for VP4 characterization. Of 21 different P genotypes, only 13 P serotypes and 3 subtypes have been identified (Estes, 2001; Hoshino et al., 2002). With some exceptions, rotavirus strains belonging to the same P serotype usually share more than 89% of amino acid identity (Gorziglia et al., 1990; Estes et al., 2001; Ciarlet et al., 1997a). The greatest VP4 sequence divergence is observed in the hypervariable region [region B, spanning from amino acid (aa) 92 to 192] of the VP4 trypsin-cleavage product VP8* that almost always correlates with VP4 serotype and subtype specificities (Larralde et al., 1991; Larralde and Gorziglia, 1992). In recent years, epidemiological surveillance to monitor the appearance of novel rotavirus antigenic types has intensified throughout the world, yielding evidence for the increasing antigenic diversity of group A rotaviruses (Rao et al., 2000; Okada and Matzumoto, 2002).

Lapine rotavirus (LRV) strains have been isolated in Canada (LRV), Japan (R-2), Italy (82/311F), and the United States (ALA, C-11, BAP-2), and those that have been characterized belong to the VP7 serotype G3 (Castrucci et al., 1985; Ciarlet et al., 1997a; Conner et al., 1988; Petric et al., 1978; Sato et al., 1982; Thouless et al., 1988) and to the VP4 serotype P11[14] (Ciarlet et al., 1997a; Hoshino et al., 2002). The P11[14] specificity is also shared by HRVs with unusual bovinelike G serotypes G6, G8, and G10, isolated in Italy, Finland, Thailand, Australia, and Egypt (Gerna et al., 1994; Urasawa et al., 1993; Palombo et al., 1999; Holmes et al., 1999), and by human G1 rotaviruses from South Africa (Mphahlele et al., 1999). With exception of the Japanese LRV strain R-2, all the LRV strains possess SGI specificity (Sato et al., 1982; Thouless et al., 1988; Ciarlet et al., 1997a, 1998). All the LRV strains analyzed so far belong to the NSP4A (KUN-like) genogroup (Ciarlet et al., 2000).

The aim of this study was to molecularly characterize the VP7, VP4, VP6, and NSP4 specificities of LRVs identified in diarrheic rabbits, experiencing prolonged episodes of mild to severe enterotyphlitis and cecal impaction, in different intensive herds in southern Italy.

Results

VP7 analysis

The basic structure of the VP7-encoding cognate gene of the Italian LRV strains 30/96, 160/01, 229/01, and 308/01 was 1062 nucleotide long, with two in-phase open reading frames (ORFs) beginning at nucleotides 49 to 51 and 136 to 138, and a single TAG codon at nucleotides 1027 to 1029, coding for a VP7 protein of 297 or 326 aa, respectively. We compared the complete deduced aa sequence for the gene encoding the VP7 of the Italian LRV strains with those of serotype G3 LRV strains isolated in different parts of the globe, serotype G3 HRV or animal rotavirus strains (Fig. 1), and with those of representative rotavirus strains from all 15 G serotypes (Table 1). A high degree of aa identity (95 to 96%) was found when the deduced aa sequences of the VP7 of the Italian LRV strains were compared to each other. The VP7 proteins of the Italian LRV strains were 94 to 97% identical to those of the G3 American LRV strains ALA, C-11, and BAP-2; 92 to 94% to that of the Japanese LRV strain R-2; and 89 to 94% to those of G3 HRV YO and rhesus rotavirus (RRV). With the remaining serotypes, aa identity ranged from 59% with avian rotavirus Ch-2 (G7) to 88% with the porcine rotavirus YM (G11). The VP7 protein

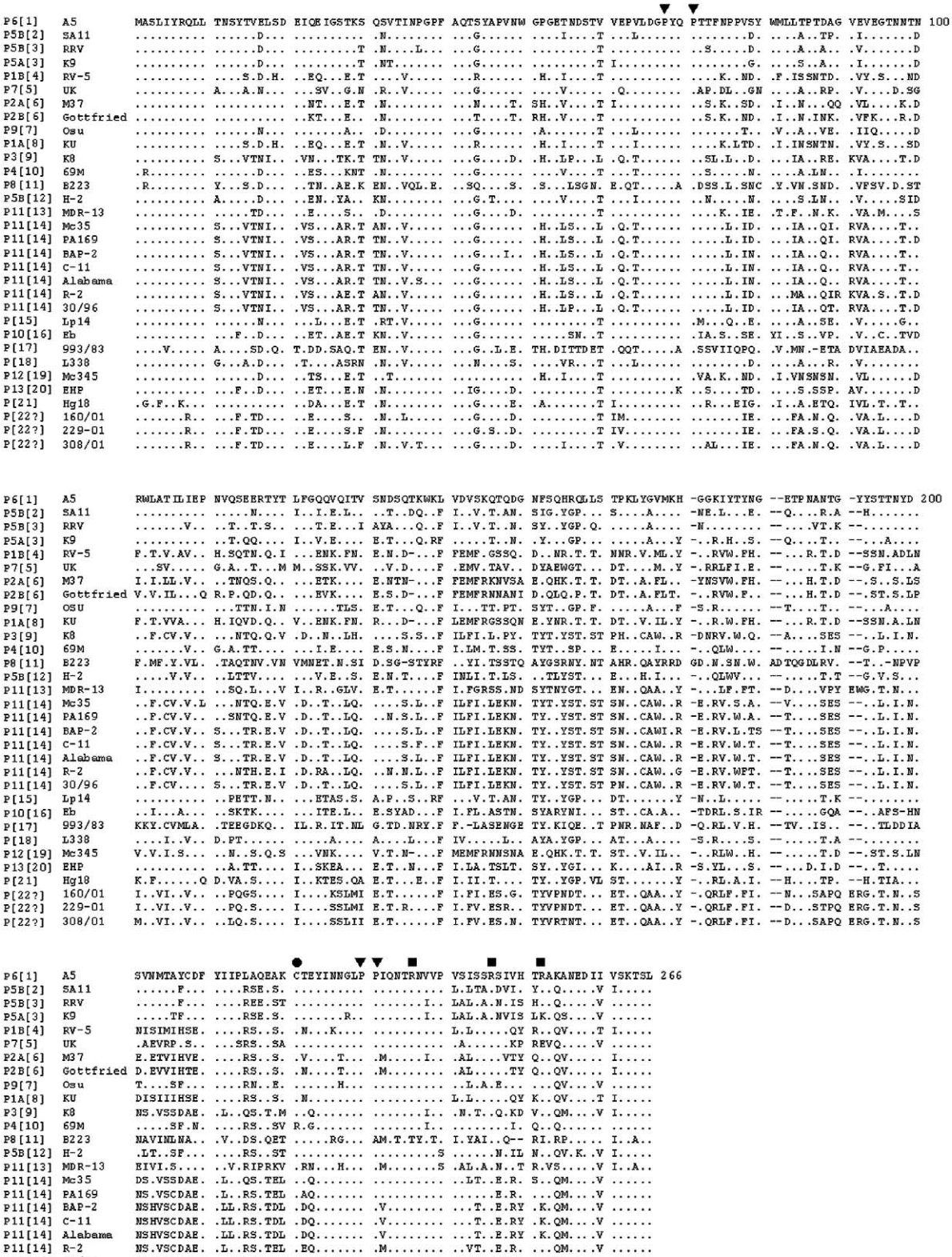

Fig. 2. Deduced amino acid sequence of the VP8* trypsin-cleavage product of the VP4 protein of the Italian LRV strains 30/96, 160/01, 229/01, and 308/01 and of a selection of strains representing the remaining P genotypes. The highly conserved cysteine (●), prolines (▼), and arginines (■) are indicated. For optimal alignment, gaps were introduced in the sequences. The Accession numbers of the VP4 sequences are listed in Table 2 The first eight residues in the sequence of the Italian LRV trains are inferred on the sequence of primer Con3 (nt 10 to 32).

Table 2
Amino acid comparison of the VP8* of Italian lapine rotaviruses with rotaviruses[a] belonging to well-established P genotypes

Strain (origin)	P genotype	P serotype	Amino acid % identity							
			VP8*				Region B (aa 92–192)			
			30/96	160/01	229/01	308/01	30/96	160/01	229/01	308/01
A5 (bovine)	1	6	55.8	58.9	58.9	57.6	49.5	46.2	47.3	47.3
SA11 (simian)	2	5B	57.2	62.7	63.1	62.7	50.6	50.5	53.8	53.7
RRV (simian)	3	5B	54.0	60.8	60.8	60.4	49.8	48.4	51.6	51.6
K9 (canine)	3	5A	54.5	62.7	62.7	60.4	47.4	52.7	53.7	53.7
RV-5 (human)	4	1B	50.8	51.3	53.4	55.8	39.8	44.1	43.0	43.0
UK (bovine)	5	7	51.3	55.4	56.3	55.8	42.0	47.3	48.4	48.4
M37 (human)	6	2A	47.6	55.9	55.9	51.3	36.6	41.9	39.8	39.8
Gottfried (porcine)	6	2B	47.6	62.2	61.7	53.9	51.6	47.3	47.3	47.3
OSU (porcine)	7	9	49.0	53.5	53.1	51.3	37.6	38.7	37.6	37.6
KU (human)	8	1A	49.4	52.2	51.7	51.7	36.5	41.9	40.8	40.8
K8 (human)	9	3	80.1	52.2	51.3	52.6	81.7	45.1	44.1	44.1
69M (human)	10	4	54.9	61.3	61.3	60.8	47.4	51.6	51.6	51.6
B223 (bovine)	11	8	34.8	34.8	33.9	33.9	22.6	24.7	24.8	24.8
H-2 (equine)	12		54.4	59.0	59.0	58.1	48.4	49.4	49.5	49.5
MDR-13 (porcine)	13		51.7	76.8	76.8	75.0	42.3	65.6	66.6	67.4
R-2 (lapine)	14	11	87.3	54.0	53.1	52.6	87.1	45.1	45.1	45.1
C-11 (lapine)	14	11	95.9	51.7	50.8	50.8	95.7	43.0	41.9	43.0
ALA (lapine)	14	11	95.9	51.3	49.9	50.3	96.8	43.0	41.9	43.0
BAP-2 (lapine)	14	11	94.6	50.3	49.4	49.4	93.5	40.9	39.8	40.9
PA169 (human)	14	11	91.4	54.4	53.5	54.0	91.4	45.1	44.1	45.1
Mc35 (human)	14	11	87.7	53.5	52.6	53.1	88.1	43.0	42.0	43.0
30/96 (lapine)	**14**	**11**[b]	—	**52.2**	**51.3**	**50.8**	—	**43.0**	**41.9**	**43.0**
Lp14 (ovine)	15		53.4	55.9	60.4	59.4	49.5	49.4	48.4	49.4
Eb (murine)	16	10	52.2	55.8	55.4	57.2	48.4	47.3	47.3	50.5
993/83 (bovine)	17		39.9	35.3	33.9	35.3	34.4	31.2	29.0	29.0
L338 (equine)	18		58.1	60.4	60.8	58.6	50.5	52.7	55.4	52.7
Mc345 (human)	19	12	51.7	53.5	54.0	54.0	41.9	45.1	46.2	46.2
EHP (murine)	20	13	54.0	60.8	60.8	59.9	50.5	52.7	52.7	52.7
Hg18 (bovine)	21		55.8	61.2	61.2	59.0	49.0	53.8	54.8	53.7
160/01 (lapine)	**22?**		**52.2**	—	—	—	**43.0**	—	—	—
229/01 (lapine)	**22?**		**51.3**	**94.5**	—	—	**41.9**	**92.5**	—	—
308/01 (lapine)	**22?**		**50.8**	**89.6**	**90.4**	—	**43.0**	**88.2**	**90.3**	—

[a] GenBank accession nos. of VP4 genes: A5 (D13395), SA11 (M23188), RRV (M18736), K9 (D14725), RV-5 (M32559), UK (M22306), M37 (L20887), Gottfried (M33516), OSU (X13190), KU (M21014), K8 (D90260), 69M (M60600), B223 (D13394), H-2 (L04638), MDR13 (L07886), R-2 (U62152), C-11 (U62150), ALA (U62149), BAP-2 (U62151), PA169 (D14724), Mc35 (D14032), Lp14 (L11599), Eb (L18992), 993/83 (D16352), L338 (D13399), Mc345 (D38054), EHP (U08424), and Hg18 (AF237665).

[b] Predicted P serotype on the basis of sequence comparison. The Italian lapine rotaviruses are in bold.

of all the Italian LRV strains had a potential N-linked glycosylation site located at aa 69 (Asn), which is also found in all serotype G3 LRV strains and tends to be conserved among most rotavirus strains (Nishikawa et al., 1989; Ciarlet et al., 1995, 1997a). The VP7 antigenic regions, A (aa 87–101), B (aa 143–152), C (aa 208–223), and F (aa 235–242) (Dyall-Smith et al., 1986; Nishikawa et al., 1989; Kobayashi et al., 1991; Ciarlet et al., 1997b) of the four Italian LRV strains and other LRVs clearly supports the inclusion of the Italian LRVs as serotype G3. Compared to the VP7 of the American or Japanese LRV strains, the VP7 of the Italian strains 160/01 and 308/01 had an aa substitution each in region A at residue 90 (Ala to Thr) and 100 (Asp to Glu), respectively, whereas those of all Italian strains had a substitution at residue 147 (Thr to Ala). Interestingly, the VP7s of strains 160/01, 229/01, and 308/01 had an aa substitution in region F at residue 238 (Asp to Asn), creating a second potential glycosylation site, which is also present in the Japanese LRV strain R-2 (Fig. 1). Thus, the sequence of the VP7 proteins of the four Italian LRV strains 30/96, 160/01, 229/01, and 308/01 confirmed their serotype G3.

VP4 analysis

The deduced aa sequences of the trypsin-cleavage VP8* product of the outer capsid protein VP4 of the four Italian LRV strains were compared with those of LRVs and HRVs that belonged to genotype P[14] (serotype P11), and with the VP8* sequences of representative strains of the 20 remaining P genotypes (Fig. 2 and Table 2). As with most rotavirus strains, the potential trypsin-cleavage sites at residues 231, 241, and 247 (Arias et al., 1996) were conserved in all four Italian LRV strains. In addition, the highly con-

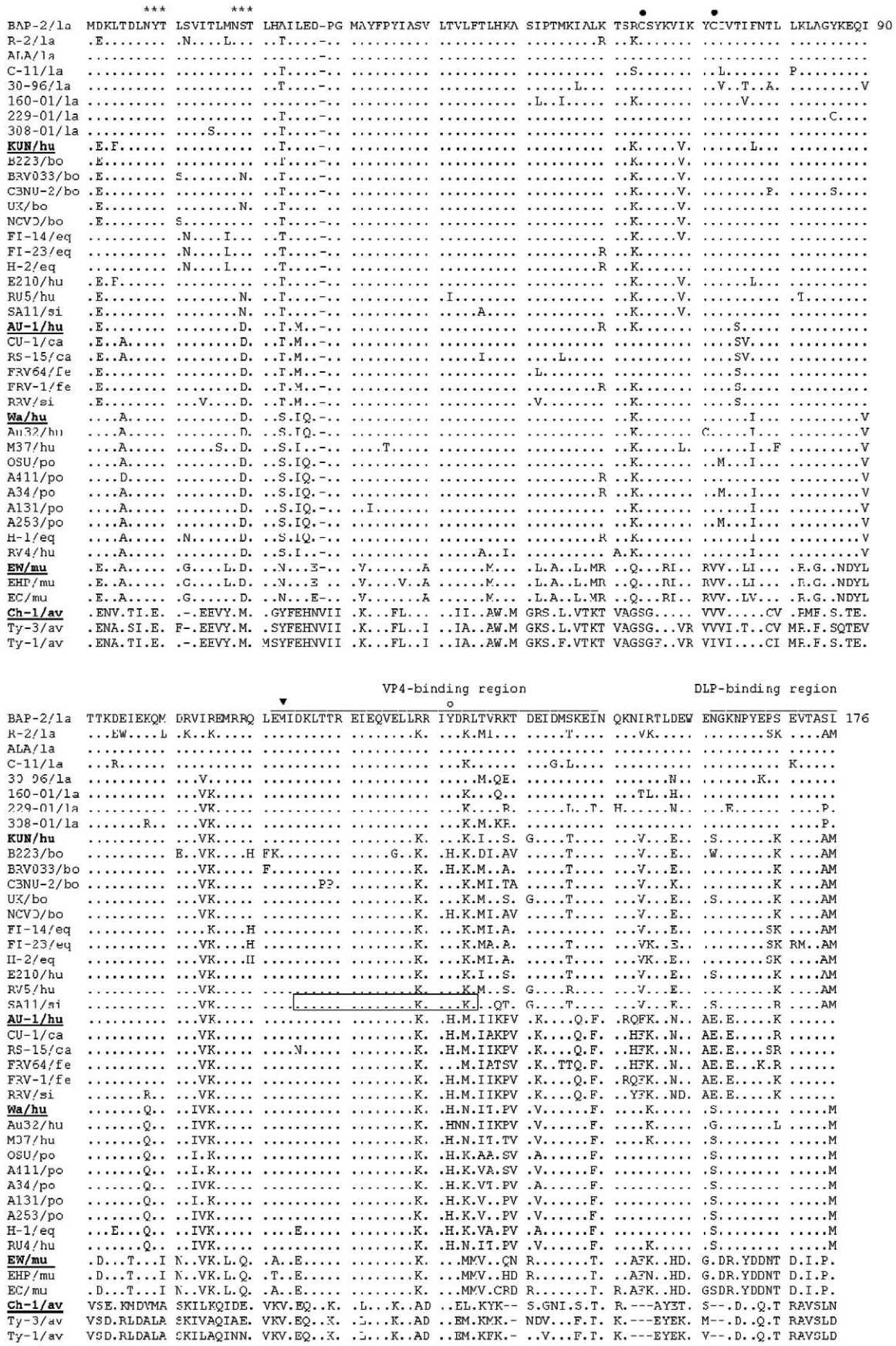

Fig. 3. Deduced amino acid sequence of the NSP4 protein of the Italian LRV strains 30/96, 160/01, 229/01, and 308/01 and of a selection of strains representing the five distinct NSP4 genotypes. The potential glycosylation sites are indicated by asterisks (*). The highly conserved cysteine (●) and methionine (▼) residues, and the putative VP4 and double-layered particle- (dlp) binding regions are shown. Also, the enterotoxic peptide of simian rotavirus SA-11 strain at aa 114–135 and tyrosine-131 (○), considered a key residue for enterotoxigenic activity, are shown (Estes, 2001). For optimal alignment, gaps were introduced in the sequences. Accession numbers of the NSP4 sequences are listed in Table 3.

Table 3
Amino acid comparison of the NSP4 of Italian lapine rotaviruses with rotaviruses[a] representative of the five NSP4 genotypes recognized to date[b]

Strain (origin)	NSP4 genotype	Amino acid % identity			
		30/96	160/01	229/01	308/01
Bap-2 (lapine)	A	93.7	93.7	94.3	88.6
R-2 (lapine)	A	88.9	85.7	86.9	89.2
ALA (lapine)	A	93.7	93.7	93.1	88.6
C-11 (lapine)	A	89.7	90.3	91.4	86.9
KUN (human)	A	90.3	88.0	88.6	89.2
B223 (bovine)	A	82.9	85.2	85.7	86.3
BRV033 (bovine)	A	86.3	88.0	88.6	89.7
CBNU-2 (bovine)	A	85.8	86.9	88.0	88.6
UK (bovine)	A	86.9	88.6	89.2	90.3
NCDV (bovine)	A	86.3	88.0	88.6	89.7
FI-14 (equine)	A	86.3	88.0	88.0	89.7
FI-23 (equine)	A	84.6	86.9	86.9	88.0
H-2 (equine)	A	85.7	88.0	88.0	89.2
E-210 (human)	A	86.3	88.6	89.2	89.7
RV5 (human)	A	86.3	88.0	88.6	89.7
SA-11 (simian)	A	86.3	89.2	88.6	89.2
AU-1 (human)	C	82.9	84.6	85.2	85.1
CU-1 (canine)	C	82.9	84.0	84.6	84.6
RS-15 (canine)	C	80.6	82.3	82.3	82.3
FRV-64 (feline)	C	82.9	83.5	84.6	83.5
FRV-1 (human)	C	81.8	83.5	84.0	84.0
RRV (simian)	C	80.6	84.6	82.9	84.0
Wa (human)	B	83.5	84.6	84.6	85.7
AU-32 (human)	B	81.8	82.9	81.8	84.7
M37 (human)	B	81.8	82.9	82.3	84.0
OSU (porcine)	B	83.5	84.0	84.0	85.7
A411 (porcine)	B	83.5	84.6	84.6	86.3
A34 (porcine)	B	83.5	84.0	84.0	85.7
A131 (porcine)	B	83.5	84.6	84.6	86.3
A-252 (porcine)	B	84.6	85.2	85.1	86.9
H-1 (equine)	B	81.8	82.9	82.9	84.6
RU-4 (human)	B	82.3	83.5	82.9	84.6
EW (murine)	D	64.7	65.2	64.7	64.1
EHP (murine)	D	64.1	64.7	64.1	63.5
EC (murine)	D	64.1	64.7	64.7	64.1
Ch-1 (avian)	E	39.6	40.1	40.1	41.3
Ty-1 (avian)	E	37.9	36.7	35.6	36.7
Ty-3 (avian)	E	37.3	37.9	37.3	38.44
30/96 (lapine)	**A**	—	**90.3**	**90.3**	**92.0**
160/01 (lapine)	**A**	**90.3**	—	**90.9**	**92.0**
229/01 (lapine)	**A**	**90.3**	**90.9**	—	**94.3**
308/01 (lapine)	**A**	**92.0**	**92.0**	**94.3**	—

[a] GenBank accession nos. of NSP4 genes: BAP-2 (AF144795), R-2 (AF144794), ALA (AF144792), C-11 (AF144793), KUN (D88829), B223 (AF144805), BRV033 (AF144804), CBNU-2 (AF166354), UK (K03384), NCDV (X06806), FI-14 (AF144803), FI-23 (AF144802), H-2 (AF144801), E210 (U59107), RV5 (U59103), SA-11 (K01138), AU-1 (D89873), CU-1 (AF144806), RS-15 (D88832), FRV64 (D88833), FRV-1 (D89874), RRV (L41247), Wa (K02032), M37 (U59109), OSU (D88831), A411 (AF144799), A34 (AF165219), A131 (AF144798), A253 (AF144797), H-1 (AF144800), RV4 (U59108), EW (U96335), EHP (U96336), EC (U96337), Ch-1 (AB065287), Ty-1 (AB065285), and Ty-3 (AB065286).

[b] The Italian lapine rotaviruses are in bold. The prototype of each genotype is in bold. The avianlike NSP4 genotype is indicated as NSP4 E, following a previous letter designation (Ciarlet et al., 2000).

served prolines at residues 68, 71, 225, and 226 and cysteine at residue 216 were maintained. The VP8* of LRV strain 30/96 showed the highest degree of aa identity (87 to 96%) with P11[14] HRVs isolated in Italy (PA169) or Thailand (Mc35), and P11[14] LRVs isolated in the United States (ALA, C-11, BAP-2) or Japan (R-2). With the remaining 20 P genotypes, the aa identity of the VP8* of the Italian LRV 30/96 ranged from 35% (Bo/B223, P8[11]) to 80% (Hu/K8, P3[9]). Conversely, the VP8* aa sequences of the noncultivable Italian LRV strains (160/01, 229/01, and 308/01), were 90 to 95% identical among each other, but with the remaining P genotypes, the aa identity ranged from 34% (Bo/B223, P8[11]) to 77% (Po/MDR-13, P[13]) (Table 2). Since it has been established that rotavirus strains that exhibit a VP4 (or VP8*) aa identity of approximately ≥89% belong to the same P genotype (Gorziglia et al., 1990), our results indicate that the Italian LRV strain 30/96 belongs to the P[14] genotype, whereas the Italian LRV strains 160/01, 229/01, and 308/01 represent a novel rotavirus P genotype.

VP6 analysis

Comparative analyses of the deduced aa sequences of the fragment of the VP6 gene, known to correlate with SG specificity (Iturriza-Gómara et al., 2002), were also performed. The corresponding VP6 region, spanning aa 281 to 350, of the Italian LRV strains revealed that all the strains belonged to genogroup I (data not shown), where strains determined as SG I [by enzyme-linked immunosorbent assay (ELISA) using the SG specific MAb 255/60] belong. Analysis of the VP6 SG-specific region of each of the Italian LRV strains showed highly conserved aa substitutions, such as Ala-305, Asn-310, Glu-315, Ser-339, and Met-342, characteristic of SG I rotaviruses (data not shown). Therefore, the four Italian LRV strains were assigned to the SG I antigenic specificity.

NSP4 analysis

At least five genetic groups of the rotavirus enterotoxin are now known (Horie et al., 1997; Ciarlet et al., 2000; Mori et al., 2002). Within NSP4 KUN-like and Wa-like genotypes, rotavirus strains isolated from rabbits, horses, cows, and pigs generally cluster according to species of origin, suggesting a constant pattern of evolution within species (Ciarlet et al., 2000). To confirm the NSP4 genotype of the LRV strains isolated in Italy, we determined the nucleotide sequence of the gene coding for the NSP4 of the four LRV strains. The fundamental structure of the NSP4 genes of all strains sequenced in this study was similar to those of other rotavirus strains sequenced previously, consisting of a 528-bp ORF, encoding a protein with a predicted size of 175 aa with the two conserved potential N-linked glycosylation sites located at aa 8 and 18. The deduced aa sequences of the NSP4 gene of all Italian LRV strains were compared with LRV strains of the NSP4A (KUN-like) genotype and other representative NSP4 sequences of genogroups A, B, C, D, and E (Fig. 3 and Table 3). The NSP4 protein of all the Italian LRV strains showed the highest aa identity (85 to

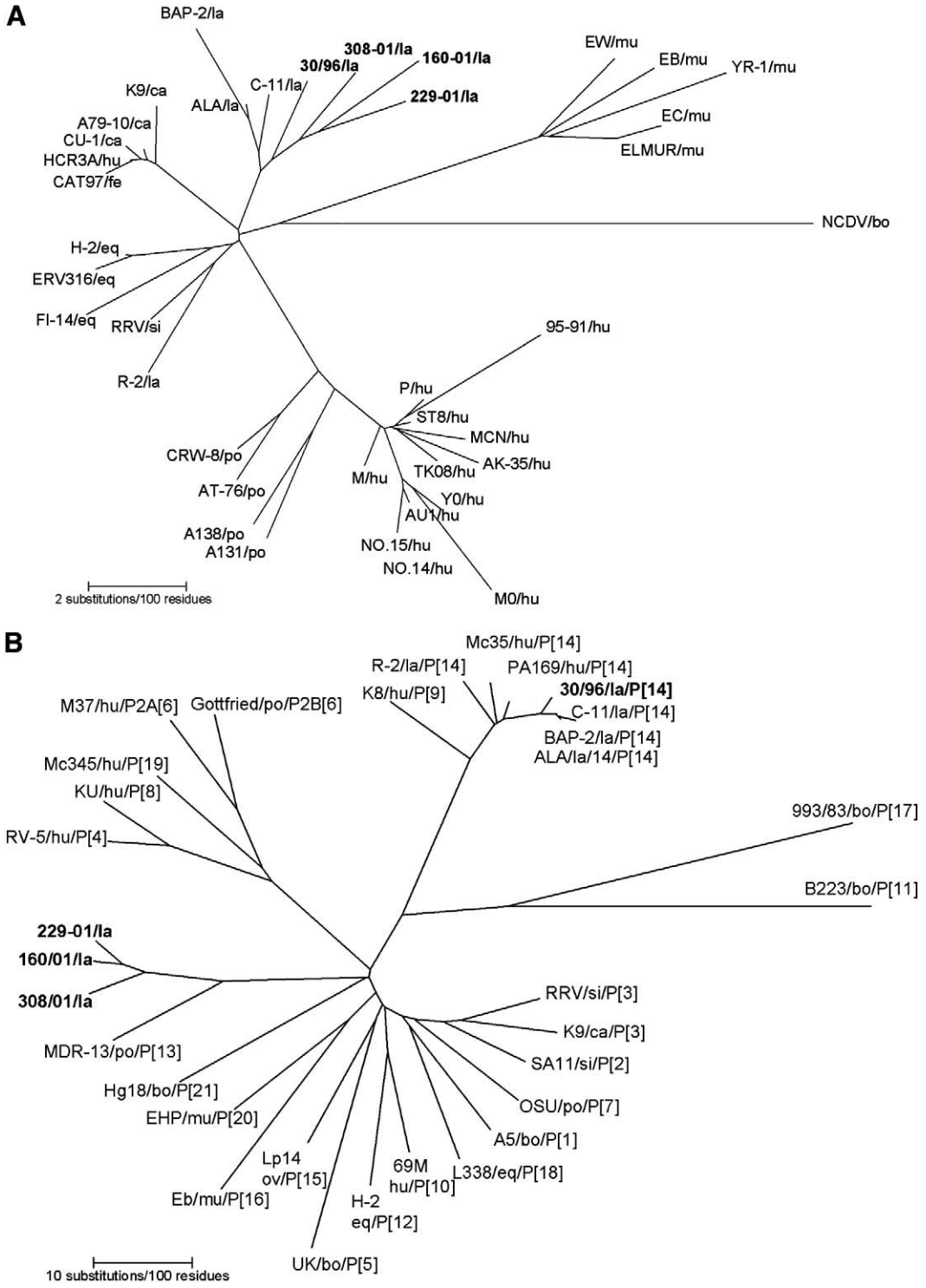

Fig. 4. Parsimony phylogenetic tree of the VP7, VP8*, and NSP4 aa sequences of the Italian LRV strains 30/96, 160/01, 229/01, and 308/01. (A) VP7 tree displaying the relationships among a selection of serotype G3 animal and HRV strains. The dendrogram is drawn to scale and rooted using the bovine strain NCDV (P6[1],G6). (B) VP8* tree displaying the relationships among strains representative of all the VP4 genotypes recognized to date. The dendrogram is drawn to scale and rooted using the avianlike bovine strain Bo/993/83 (P[17],G7). (C) NSP4 tree displaying the relationships among a selection of animal and HRV strains representative of the five NSP4 genetic groups. The dendrogram is drawn to scale and rooted using the murine strains. The branch of the avian strains Ch-1, Ty-1, and Ty-3 (P[17],G7) is out of scale. The prototypes of the NSP4 genotypes (strains KUN, Wa, Au-1, and EW) are in bold and underlined. Abbreviations: si (simian), la (lapine), eq (equine), po (porcine), ca (canine), fe (feline), bo (bovine), mu (murine), and hu (human). The Italian LRV strains are shown in bold type.

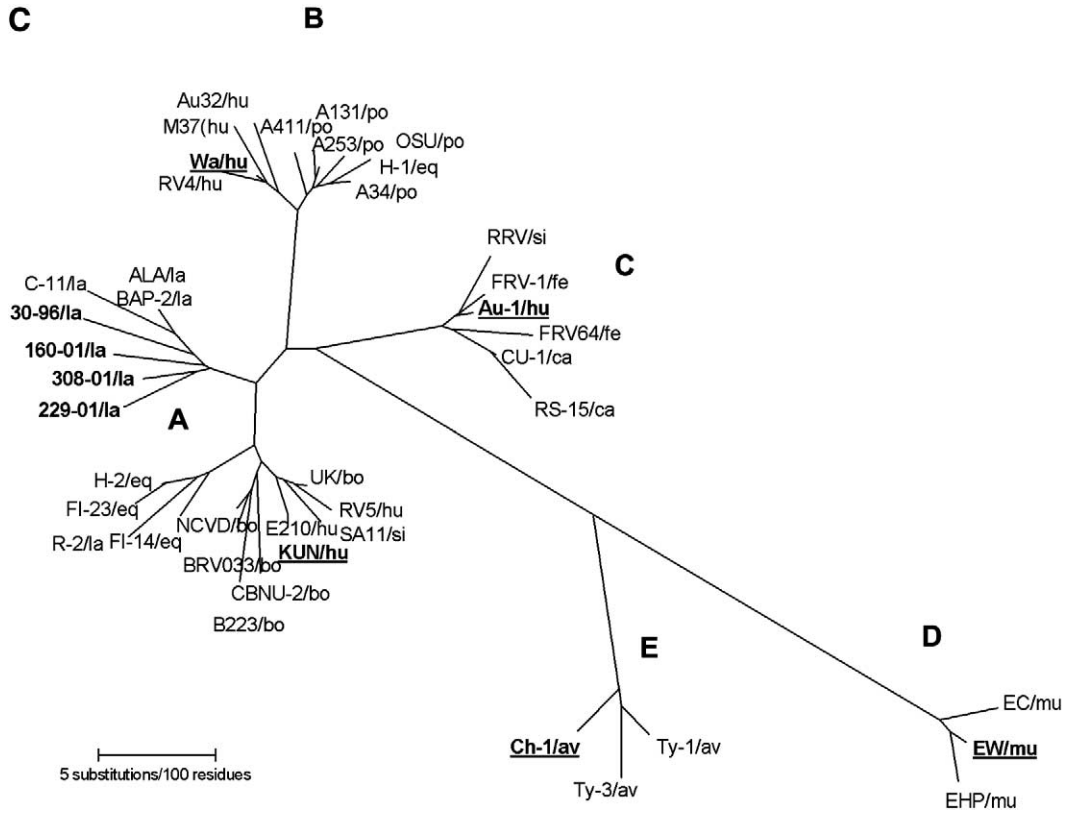

Fig. 4 (*continued*)

95%) to those of the LRV strains, with a few scattered aa substitutions throughout the NSP4 protein with respect to American or Japanese LRVs and the hypervariable region (aa 135 to 141) associated with altered virulence in mice (Ciarlet et al., 2000). Of interest, based on the aa percentage identitities, the LRV NSP4s are almost as genetically distant from nonlapine NSP4s of geonogroup A (KUN-like) as they are from those in the genogroup B (Wa-like).

Phylogenetic relationships in the VP7, VP8(VP4), and NSP4 proteins*

Parsimony phylogenetic analyses of the deduced aa sequences of the VP7, VP8*, and NSP4 genes of the Italian LRV strains 30/96, 160/01, 229/01, and 308/01 provided a molecular basis for their relatedness to previously characterized LRV strains (Fig. 4). The phylogenetic relationship of the VP7 of the four Italian LRV strains to those of G3 LRV, HRV, or other rotavirus strains confirmed their lapine origin (Fig. 4A). In the phylogenetic tree, the VP8* of the LRV strain 30/96 clustered with P11[14] LRV or HRV strains as expected, whereas the noncultivable LRV strains 160/01, 229/01, and 308/01 were placed on a clearly distinct branch, close to that of the P[13] porcine strain MDR-13 (Fig. 4B). Therefore, LRV strains 160/01, 229/01, and 308/01 qualify as a new P genotype. The deduced NSP4 aa sequences of the four Italian LRV strains clustered together with the LRV strains belonging to the NSP4A, KUN-like, genotype (Fig. 4C), establishing their close relationship with other LRV strain isolated from other parts of the world.

Discussion

All the LRV strains detected worldwide and analyzed to date form a highly conservative group, with all the strains characterized possessing a serotype G3 VP7 type associated with a single P serotype, P11[14], and a single NSP4 genotype, KUN-like (Castrucci et al., 1985; Conner et al., 1988; Petric et al., 1978; Sato et al., 1982; Thouless et al., 1988; Ciarlet et al., 1997a, 2000; Hoshino et al., 2002). In addition, with the exception of the Japanese LRV strain R-2, that belongs to the VP6 SG II, all LRVs have been shown to possess a SG I VP6 specificity (Sato et al., 1982; Thouless et al., 1988; Ciarlet et al., 1997a).

In this study, we characterized molecularly the VP7, VP4, VP6, and NSP4 genes of LRVs identified in rabbits in different rabbitries in the Basilicata, northern Puglia, and southern Puglia regions of Italy. All the Italian LRV strains analyzed displayed a VP7 of serotype G3 specificity. Although the G3 VP7 type is shared by rotaviruses from a broad spectrum of mammalian species, including humans (Nishikawa et al., 1989), it has been possible to identify species-specific sequences in the VP7 protein, as well as to

show a species-specific segregation of the VP7 protein by phylogenetic analysis (Nishikawa et al., 1989; Ciarlet et al., 1995; Martella et al., 2001). Accordingly, the VP7 proteins of the Italian LRV strains were conserved with those of the American or Japanese LRV strains (Nishikawa et al., 1989; Ciarlet et al., 1997a). However, the VP7 proteins of all the Italian LRV strains had a unique aa change at position 147 (Thr → Ala), within the VP7 hypervariable region B, considered critical for both serotype and monotype specificity (Dyall-Smith et al., 1986; Coulson and Kirkwood, 1991). Further studies will be needed to determine if the change at position 147 in the Italian VP7 proteins correlates with any antigenic changes with respect to the previously characterized LRV strains.

Similarly to VP7, the NSP4 proteins of the KUN- and Wa-like genotypes contain species-specific sequences allowing rotavirus strains isolated from animals to generally cluster according to species of origin by phylogenetic analysis (Ciarlet et al., 2000). In our analysis, the NSP4 proteins of all four Italian LRV strains clustered together with the American and Japanese LRV strains belonging to the KUN-like NSP4 genotype, providing further evidence that the strains analyzed are of lapine origin. The clear species-specific pattern of segregation of LRVs in the NSP4 phylogenetic, as well as the genetic distance of LRV NSP4s between those belonging to the genogroup A and B, suggest that the NSP4 of LRVs are genetically in an intermediate position within these two genogroups. However, molecular analysis of additional NSP4s of lapine origin would help classify LRV NSP4s into a separate genogroup or as a subgroup within the genogroup A. Also, the identification that the Italian LRV strains possess a SG I specificity, is consistent with the animal and lapine origin of the strains.

We also identified, for the first time, a P[14] rotavirus strain (30/96) of animal origin in Italy, where, in the mid-1990s, P11[14] human rotaviruses had been first observed (Gerna et al., 1994). The LRV strain 30/96, successfully adapted to cell culture, was isolated in 1996 in the region of Puglia, southern Italy, but no additional P[14] LRVs were detected during our study. Nevertheless, the VP4 specificity of the other Italian LRV strains, 160/01, 229/01, and 308/01, identified in 2001, could not be assigned to any of the existing P genotypes. The significantly low aa sequence identity of the VP4 trypsin-cleavage fragment VP8*, whose sequence divergence correlates with the P genotype (Larralde et al., 1991; Larralde & Gorziglia, 1992) of the LRV strains 160/01, 229/01, and 308/01 to all other established P genotypes, indicates that these strains belong to a novel VP4 genotype. All the LRVs identified in 2001 were detected in young rabbits with clinical disease in different and distant areas of southern Italy, indicating that, after their onset, they quickly spread throughout the Italian territories, possibly due to commercial movements of breeders or slaughtering of animals. Because there are no epidemiological data on the prevalence of rotavirus G or P types in rabbits in Italy since the mid-1980s, and the P type of the Italian LRV strain 82/311F (isolated in 1982) was never determined (Castrucci et al., 1985), it is difficult to speculate when this novel P genotype emerged in the rabbit population. Still, the consistent intragenotype variability observed (between 5.5 to 10.4%) is suggestive of independent diversification of the strains identified in the three different rabbitries in 2001, as well as of a wide distribution. Extensive analysis on rotaviruses from several rabbit herds is currently ongoing in our laboratories to assess whether the P[14] genotype has been replaced by this novel VP4 type and to elucidate the current relative distribution of the two VP4 types among rabbit rotaviruses in Italy.

The rotavirus spike protein VP4 is responsible for a number of important biological functions, such as the enhancement of infectivity by proteolytic cleavage into VP8* and VP5*, hemagglutination, restriction of growth in cell culture, virulence, initial virus attachment to cells, protease sensitivity associated to plaque formation, and host range restriction (Arias et al., 1996; Bridger et al., 1992; Espejo et al., 1981; Greenberg et al., 1983; Kalica et al., 1983; Offit et al., 1986; Ciarlet et al., 1988, 2002). The identification of serotype G3 LRV strains associated with a VP4 genotype different from P[14], whereas providing additional evidence for the increasing genetic/antigenic diversity of group A rotaviruses in nature, is also important from the perspective of understanding the molecular basis of the refractivity of some rotavirus strains to grow in cell culture. In contrast to the P[14] LRV strain 30/96, we failed to adapt the LRV strains 160/01, 229/01, and 308/01, belonging to the novel P genotype, to grow in cell culture. The different VP4 types of the strain of 1996 and those of 2001 could explain the poor permissiveness of the MA-104 and primary rabbit kidney cells to infection by the LRV strains 160/01, 229/01, and 308/01 (data not shown). Whether the different VP4 specificities are also responsible for different pathogenic properties and/or adaptive advantage in the rabbit host should be better addressed in future studies.

In conclusion, by analyzing the VP7, VP8*(VP4), VP6, and NSP4 genes of LRV strains in Italy, we identified that all the Italian LRV strains are SG I and belong to the VP7 serotype G3 and the KUN-like NSP4 genotype. One LRV strain, isolated in the mid-1990s, belonged to the established P genotype P[14], like all LRV strains characterized to date, while the other three strains, identified in 2001, belonged to a novel VP4 type, which allow us to tentatively propose as the P[22] genotype. Because the emergence of P11[14] rotaviruses in humans has been speculated to be the result of natural reassortment in either cattle or humans after heterologous infection with rabbit P11[14] rotaviruses (Ciarlet et al., 1997a), the potential spread of this novel lapine VP4 type to human rotaviruses should also be evaluated. Characterization of additional nontypeable human and animal strains may reveal the distribution of this novel VP4 type and help to clarify its species of origin. A better understanding of the rotavirus epidemiology will contribute to the optimization of current vaccines and prevention programs

of rotavirus diarrhea in humans and animals. Furthermore, precise evaluation of antigenic or molecular diversity among domestic animal herd populations is of critical importance for the development of an effective vaccine because animal rotaviruses might be involved in interspecies transmission or reassortment in humans.

Materials and methods

Origin of samples and viruses

LRV strain 30/96 was isolated in 1996 from the rotavirus-positive intestinal contents of a diarrheic rabbit, obtained from an intensive rabbitry in Puglia, southern Italy, and was adapted to cell culture on MA-104 cells by nine serial passages. During the course of the 2001 study on the incidence of rotavirus infections in rabbits, a total of 29 fecal samples were collected from 35- to 45-day-old rabbits affected either with catarrhal enterotyphlitis or cecal impaction rabbits in three different intensive herds of southern Italy (Basilicata, northern Puglia, and southern Puglia), about 100–150 km apart. Rotavirus-positive samples were determined by an immunochromatographic assay (Rotascreen Dipstick, Microgen Bioproducts, Camberley, UK) according to the manufacturer's protocol. Five of 10 (50%), 4 of 9 (44%), and 3 of 10 (30%) of the fecal samples, obtained from the rabbitries in the Basilicata, northern Puglia, and southern Puglia regions, respectively, were identified as containing group A rotaviruses (data not shown). Despite numerous attempts on both MA-104 cells and primary fetal rabbit kidney cells, it was not possible to adapt any of the 2001 lapine strains to cell culture (data not shown).

RNA extraction and PCR amplification of the VP7, VP4, VP6, and NSP4

Viral dsRNA was extracted by adsorption on cellulose CF11 (Wilde et al., 1990) from either infected cells (strain 30/96) or directly from the feces for noncultivatable LRV strains (160/01, 229/01 and 308/01). Determination of the VP7 specificities of all LRV strains was achieved by sequence analysis of the full-length VP7 gene, whereas that of VP4 was obtained by sequencing the entire VP8* trypsin-cleavage product of VP4 and the N-terminus of the VP5* trypsin-cleavage product of VP4. The full-length VP7 gene [1062 basepairs (bp)] was reverse transcribed and amplified using the primer pair Beg9/End9 (Gouvea et al., 1990). The 876-bp VP4 fragment (VP8* and N-terminus of VP5*) was reverse transcribed and amplified with the primer pair Con2/Con3 (Gentsch et al., 1992). The VP6 genogroup, predictive of the VP6 SG specificity, was determined by amplification of a 379-bp fragment, spanning from aa 281 to 350, by using the primer pair VP6-F/VP6-R (Iturriza-Gómara et al., 2002). The nearly full length dsRNA segment encoding for the NSP4 protein was reverse transcribed and amplified using primer pair 10Beg16/10End722 (Lee et al., 2000).

Sequence and phylogenetic analysis

The sequences of the VP7, VP8*, VP6, and NSP4 genes of the Italian LRV strains were obtained directly by sequencing of the amplicons by the dideoxynucleotide chain termination method using an ABI-377 automatic DNA sequencer (Perkin–Elmer Applied Biosystems, Foster City, CA). For direct sequencing, four distinct PCR amplicons of each sample were analyzed and a consensus was determined. Because the sequences of LRVs analyzed within each rabbitry in 2001 were almost identical to each other (data not shown), the LRV strains circulating within a single rabbitry were most likely the same. Therefore, we considered the sequences of each of the noncultivable LRV strains, collected from each of the three different rabbitries in the Basilicata, northern Puglia, and southern Puglia regions, as single LRV strains named 160/01, 229/01 and 308/01, respectively. Moreover, the VP8*-encoding fragment of strain 160/01 was cloned into the vector pCRT7/NT-TOPO (Invitrogen BV, Groningen, The Netherlands) following the manufacturer's protocol and the sequence was determined using three plasmid clones. Additional internal primers were chosen on the basis of the sequences obtained when necessary to complete the analysis. Sequence data reported in this work have been deposited in the GenBank data library under accession numbers AF528204, AF528202, AF528203, and AF528201 for the VP7; AF526376, AF526374, AF526375, and AF526373 for the VP8*; and AF533534, AF533535, AF533536, and AF533537 for the NSP4 of lapine rotavirus strains 30/96, 160/01, 229/01, and 308/01, respectively.

Sequence alignment was done using Clustal W (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar et al., 2001) and PAUP version 4.0b (Swofford et al., 1998). VP7, VP4, and NSP4 parsimony trees were elaborated using a heuristic algorithm and supplying a statistical support by bootstrapping over 100 replicates.

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References

Arias, C.F., Romero, P., Alvarez, V., López, S., 1996. Trypsin activation pathway of rotavirus infectivity. J. Virol. 70, 5832–5839.

Ball, J.M., Tian, P., Zeng, C.Q.-Y., Morris, A.P., Estes, M.K., 1996. Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. Science. 272, 101–104.

Bridger, J., Burke, B., Beards, G.M., Desselberger, U., 1992. The pathogenicity of two porcine rotaviruses differing in their in vitro growth characteristics and genes 4. J. Gen. Virol. 73, 3011–3015.

Castrucci, G.M., Ferrari, M., Frigeri, V., Cilli, V., Perecca, L., Donelli, G., 1985. Isolation and characterization of cytopathic strains of rotavirus from rabbits. Arch. Virol. 83, 99–104.

Ciarlet, M., Estes, M.K., Conner, M.E., 1997a. Comparative amino acid sequence analysis of the outer capsid protein VP4 from four lapine rotavirus strains reveal identity with genotype P[14] human rotavirus. Arch. Virol. 142, 1059–1069.

Ciarlet, M., Hoshino, Y., Liprandi, F., 1997b. Single point mutation may affect the serotype reactivity of G11 porcine rotavirus strains: a widening spectrum? J. Virol. 71, 8213–8220.

Ciarlet, M., Ludert, J.E., Liprandi, F., 1995. Comparative amino acid sequence analysis of the major outer capsid protein (VP7) of porcine rotaviruses with G3 and G5 serotype specificities isolated in Venezuela and Argentina. Arch. Virol. 140, 437–451.

Ciarlet, M., Liprandi, F., Conner, M.E., Estes, M.K., 2000. Species specificity and interspecies relatedness of NSP4 genetic groups by comparative NSP4 analyses of animal rotaviruses. Arch. Virol. 145, 371–383.

Ciarlet, M., Estes, M.K., Barone, C., Ramig, R.F., Conner, M.E., 1998. Analysis of host range restriction determinants in the rabbit model: comparison of homologous and heterologous rotavirus infections. J. Virol. 72, 2341–2351.

Ciarlet, M., Ludert, J.E., Iturriza-Gómara, M., Liprandi, F., Gray, J.J., Desselberger, U., Estes, M.K., 2002. Initial interaction of rotavirus strains with *N*-acetylneuraminic (sialic) acid residues on the cell surface correlates with VP4 genotype, not species of origin. J. Virol. 76, 4087–4095.

Conner, M.E., Estes, M.K., Graham, D.Y., 1988. Rabbit model of rotavirus infection. J. Virol. 62, 1625–1633.

Coulson, B., Kirkwood, C., 1991. Relation of VP7 amino acid sequence to monoclonal antibody neutralization of rotavirus and rotavirus monotype. J. Virol. 65, 5968–5974.

Cunliffe, N.A., Woods, P.A., Leite, J.P., Das, B.K., Ramachandran, M., Bhan, M.K., Hart, C.A., Glass, R.I., Gentsch, J.R., 1997. Sequence analysis of NSP4 gene of human rotavirus allows classification into two main genetic groups. J. Med. Virol. 53, 41–50.

Dyall-Smith, M.L., Lazdins, I., Tregear, G.W., Holmes, I.H., 1986. Location of the major antigenic sites involved in rotavirus serotype-specific neutralization. Proc. Natl. Acad. Sci. USA 83, 3465–3468.

Espejo, R.T., López, S., Arias, V., 1981. Structural polypeptides of simian rotavirus SA11 and the effect of trypsin. J. Virol. 37, 156–160.

Estes, M.K., 2001. Rotaviruses and their replication, in: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Strais, S.E. (Eds.), Fields Virology, 4th ed. Lipincott William & Wilikins, Philadelphia, pp. 1747–1785.

Gentsch, J.R., Glass, R.I., Woods, P., Gouvea, V., Gorziglia, M., Flores, J., Das, B.K., Bhan, M.K., 1992. Identification of group rotavirus gene 4 types by polymerase chain reaction. J. Clin. Microbiol. 30, 1365–1373.

Gerna, G., Sears, J., Hoshino, Y., Steele, A.D., Nakagomi, O., Sarasini, A., Flores, J., 1994. Identification of a new VP4 serotype of human rotaviruses. Virology 200, 66–71.

Gorziglia, M., Larralde, G., Kapikian, A.Z., Chanock, R.M., 1990. Antigenic relationships among human rotaviruses as determined by outer capsid protein VP4. Proc. Natl. Acad. Sci. USA. 87, 7155–7159.

Gouvea, V., Glass, R.I., Woods, P., Taniguchi, K., Clark, H.F., Forrester, B., Fang, Z.-Y., 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J. Clin. Microbiol. 28, 276–282.

Greenberg, H.B., Flores, J., Kalika, A.R., Wyatt, R.G., Jones, R., 1983. Gene coding assignments for growth restriction neutralization and subgroup specificities of the Wa and DS-1 strains of human rotavirus. J. Gen. Virol. 64, 313–320.

Holmes, J.L., Kirkwood, C.D., Gerna, G., Clemens, J.D., Rao, M.R., Naficy, A.B., Abu-Elyazeed, R., Savarino, S.J., Glass, R.I., Gentsch, J.R., 1999. Characterization of unusual G8 rotavirus strains isolated from Egyptian children. Arch. Virol. 144, 1381–1396.

Horie, Y., Masamune, O., Nakagomi, O., 1997. Three major alleles of rotavirus NSP4 proteins identified by sequence analysis. J. Gen. Virol. 78, 2341–2346.

Hoshino, Y., Jones, R.W., Kapikian, A.Z., 2002. Characterization of neutralization specificities of outer capsid spike protein VP4 of selected murine, lapine, and human rotavirus strains. Virology 299, 64–71, doi: 10.1006/viro.2002.1474.

Kalika, A.R., Flores, J., Greenberg, H.B., 1983. Identification of the rotaviral gene that codes for hemagglutination and protease-enhanced plaque formation. Virology 125, 194–205.

Iturriza Gómara, M., Wong, C., Blome, S., Desselberger, U., Gray, J., 2002. Molecular characterization of VP6 genes of human rotavirus isolates: correlation of genogroups with subgroups and evidence for independent segregation. J. Virol. 76, 6596–6601.

Kirkwood, C., Palombo, E.A., 1997. Genetic characterisation f the rotavirus nonstructural protein NSP4. Virology 236, 258–265.

Kobayashi, N., Taniguchi, S., Urasawa, S., 1991. Analysis of the newly identified neutralization epitopes on VP7 of human rotavirus serotype 1. J. Gen. Virol. 72, 117–124.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics 17, 1244–1245.

Larralde, G., Gorziglia, M., 1992. Distribution of conserved and serotype-specific epitopes on the VP8* subunit of rotavirus VP4. J. Virol. 72, 117–124.

Larralde, G., Kapikian, L.B., Gorziglia, M., 1991. Serotype-specific epitope(s) present on the VP8* subunit of rotavirus VP4. J. Virol. 65, 3213–3218.

Lee, C.-N., Wang, Y.-L., Kao, C.-L., Zao, C.-L., Lee, C.-Y., Chen, H.-N., 2000. NSP4 gene analysis of rotaviruses recovered from infected children with and without diarrhea. J. Clin. Microbiol. 38, 4471–4477.

Martella, V., Pratelli, A., Greco, G., Gentile, M., Fiorente, P., Tempesta, M., Buonavoglia, C., 2001. Nucleotide sequence variation of the VP7 gene of two G3-type rotaviruses isolated from dogs. Virus Res. 74, 17–25.

Mori, Y., Borgan, M.A., Ito, N., Sugiyama, M., Minamoto, N., 2002. Diarrhea-inducing activity of avian rotavirus NSP4 glycoproteins, which differ greatly from mammalian rotavirus NSP4 glycoproteins in deduced amino acid sequence, in suckling mice. J. Virol. 76, 5829–5834.

Mphahlele, M.J., Peenze, I., Steele, A.D., 1999. Rotavirus strains bearing the VP4P[14] genotype recovered from South African children with diarrhoea. Arch. Virol. 144, 1027–1034.

Nishikawa, K., Hoshino, Y., Taniguchi, K., Green, K.Y., Greenberg, H.B., Kapikian, A.Z., Chanock, R.M., Gorziglia, M., 1989. Rotavirus VP7 neutralization epitopes of serotype 3 strains. Virology 171, 503–515.

Okada, N., Matsumoto, Y., 2002. Bovine rotavirus G and P types and sequence analysis of the VP7 gene of two G8 bovine rotavirus from Japan. Vet. Microbiol. 84, 297–305.

Offit, P.A., Blavat, G., Greenberg, H.B., Clark, H.F., 1986. Molecular basis of rotavirus virulence: role of gene segment 4. J. Virol. 57, 46–49.

Palombo, E.A., Clark, R.C., Bishop, R.F., 1999. Characterization of a "European-like" serotype G8 human rotavirus isolated in Australia. J. Med. Virol. 60, 56–62.

Petric, M., Middletown, P.J., Grant, C., Tam, J.S., Hewitt, C.M., 1978. Lapine rotavirus: preliminary study on epizoology and transmission. Can. J. Comp. Med. 42, 143–147.

Rao, C.D., Gowda, K., Reddy, B.S.Y., 2000. Sequence analysis of VP4 and VP7 genes of nontypeable strains identifies a new pair of outer capsid

proteins representing novel P and G genotypes in bovine rotaviruses. Virology 276, 104–113, doi: 10.1006/viro.2000.0472.

Sato, K., Inaba, Y., Miura, Y., Tokuhisa, S., Matumoto, M., 1982. Antigenic relationships between rotaviruses from different species as studied by neutralization and immunofluorescence. Arch. Virol. 73, 45–50.

Swofford, D.L., 1998. PAUP*, phylogenetic analysis using parsimony (*and other methods), version 4.0b8. Sinauer, Sunderland, Mass.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Thouless, M.E., DiGiacomo, R.F., Deeb, B.J., Howard, H., 1988. Pathogenicity of rotavirus in rabbit. J. Clin. Microbiol. 26, 943–947.

Urasawa, T., Taniguchi, K., Kobayashi, N., Mise, K., Hasegawa, A., Yamazi, Y., Urasawa, S., 1993. Nucleotide sequence of VP4 and VP7 genes of a unique human rotavirus strain Mc35 with subgroup I and serotype 10 specificity. Virology 195, 766–771.

Wilde, J., Eiden, J., Yolken, R., 1990. Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse transcriptase and polymerase chain reactions. J. Clin. Microbiol. 28, 1300–1307.